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Cyclic AMP-dependent phosphorylation and regulation of the cardiac dihydropyridine-sensitive Ca channel

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A polyclonal antibody, CR2, prepared using the C-terminal peptide of the α1 subunit of the rabbit cardiac DHP-sensitive Ca channel, specifically immunoprecipitated the [^{3}H]PN200-110-labeled Ca channel solubilized from cardiac microsomes. The antibody recognized 250 and 200-kDa cardiac microsomal proteins as determined by immunoblotting, and cAMP-dependent protein kinase phosphorylated the 250-kDa, but not the 200-kDa protein in vitro. CHO cells, transfected with the cardiac α1 subunit cDNA carried by an expression vector, synthesized a 250-kDa protein which was recognized by CR2. Adding db-cAMP or forskolin to the transformed CHO cells induced phosphorylation of the 250-kDa protein and stimulated the DHP-sensitive Ba current under patch-clamp conditions. These results suggested that the cardiac DHP-sensitive Ca channel was regulated by cAMP-dependent phosphorylation of the α1 subunit.

Cardiac Ca channel; Dihydropyridine; cAMP-dependent phosphorylation; Ca channel modulation

1. INTRODUCTION

Stimulation of Ca^{2+} influx through the DHP-sensitive Ca channel into cardiac muscle is essential for the isotropic effect caused by β-adrenergic drugs [1]. The activation of the β-adrenergic receptor induces the elevation of intracellular cAMP followed by the activation of PKA [2]. There is much evidence indicating that the PKA-mediated phosphorylation participates in the stimulation of the Ca current [3,4], however, phosphoproteins involved in this regulation remain to be identified.

The DHP-sensitive Ca channel has been purified from skeletal muscle and is composed of α1, α2, β, γ and δ subunits [5]. All subunits have been investigated by cDNA cloning and their primary amino-acid sequences have been determined [6–9]. The α1 and β subunits of the purified skeletal muscle Ca channel are phosphorylated by PKA accompanied by stimulation of the Ca channel function [5,10–12]. The α1 subunit was further shown to be phosphorylated in Ser-687 by PKA [13]. The cardiac DHP-sensitive Ca channel has been partially purified and contains α1 and α2 subunits, however the precise subunit composition has not been established [14–16]. Several reports have shown that the α1 subunit in partially purified cardiac Ca channels is not phosphorylated by PKA [14–16] and its corresponding Ser-687 region of the skeletal muscle α1 subunit is not a phosphate acceptor [17]. From the amino acid sequence, there are several possible sites of PKA mediated phosphorylation in the C-terminal region of the cardiac α1 subunit [17]. The molecular mass of the α1 subunit of partially purified cardiac Ca channel is about 200 kDa [14–16] and is much smaller than that deduced from the amino acid sequence (M_r 242,771), leading to the notion that the C-terminal region containing potential phosphorylation sites is cleaved from the α1 subunit during partial purification. We investigated the phosphorylation of the α1 subunit in rabbit heart and CHO cells expressing the α1 subunit and the effect of db-cAMP and forskolin on the Ca current in CHO cells.

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Abbreviations: db-cAMP, dibutyryl cAMP; CHO, Chinese hamster ovary; DHP, dihydropyridine; I_{Ba}, Ba current; PKA, cAMP-dependent protein kinase; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2, 6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate.

2. EXPERIMENTAL

2.1. *Preparation of the polyclonal antibody, CR2*

The 2.0-kb *Bgl*II (4892)/*Bam*HI (vector) fragment from pCARD3 [17] was cloned into the *Bam*HI site of pAR3038 [18] to yield pCAS9, in which the cDNA insert was positioned downstream of and in the same orientation as the φ10 promoter for T7 RNA polymerase. The generated expression plasmid pCAS9 contains the 549 amino acid-coding region of the C terminus in the α1 subunit of the rabbit cardiac Ca channel, fused in-frame, with the translation initiation codon provided on the pAR3038. *E. coli* BL21 transformed with pCAS9 was cultured and the fusion protein obtained as an insoluble pellet [19]. The fusion protein (0.22 mg per rabbit) was further purified by SDS-PAGE. The fusion protein, which located on the gel by copper chloride staining [20], was fragmented and injected into multiple sites on

the back of rabbit with Freund's complete adjuvant. Immunization without adjuvant was repeated every 3–4 weeks and blood was taken 7 to 10 days after each booster.

2.2. *Isolation of CHO cells expressing the α1 subunit of rabbit cardiac DHP-sensitive Ca channel*

The 7.0-kb *Hin*dIII fragment containing the entire protein-coding sequence of the cardiac α1 subunit of DHP-sensitive Ca channel from pCARD1 [17] was cloned into the *Hin*dIII site of pKNH [21] to yield pCCAR. CHO cells were transfected with *Pvu*I-cleaved pCCAR. Clones CCAR3217 and CCAR2823 were isolated by screening G418-resistant clones by RNA blotting using the cDNA of cardiac α1 subunit as the probe. Cells were maintained in MEM α medium (Gibco, no. 410-1900) supplemented with 10% calf serum.

2.3. *Membrane preparation*

Cardiac membranes were prepared from rabbit ventricles [22]. CHO and CCAR cells were homogenized in NEH solution (125 mM NaCl, 1 mM EDTA, and 25 mM HEPES-Tris, pH 7.4) by sonication. The homogenates were centrifuged at 540,000 × *g* for 10 min at 4°C and the membrane pellet was washed once with 0.5 M KCl/NEH, resuspended in NEH and stored at −80°C. Protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 1 mM 1,10-phenanthroline, 1 μg/ml antipain and 1 μg/ml leupeptin) were added throughout the preparation.

2.4. *Immunoprecipitation and immunoblotting of the Ca channel proteins with CR2*

The [^{3}H]PN200-110-labeled rabbit cardiac Ca channels were precipitated as previously described [23]. Immunoblotting was performed as described in [24] except using a semi-dry transblotting apparatus, Transblot SD (Bio-Rad) and a blotting solution containing 20% methanol, 48 mM Tris, 39 mM glycine, and 1.3 mM SDS at pH 9.2. The ECL Western blotting detection reagent (Amersham) was used for detection.

2.5. *Phosphorylation of the α1 subunit in vitro and in vivo*

Membranes were solubilized with 1% digitonin in NEH and the Ca channel proteins were immunoprecipitated with CR2 or control rabbit IgG and phosphorylated with a catalytic subunit of PKA (35.7 U) as described in [14]. The phosphorylated proteins were analyzed by SDS-PAGE and a Fuji Bioimage-analyzer BAS 2000 (Fuji Photo Film Co.) using an imaging plate [25].

Phosphorylation of the α1 subunit in vivo was measured by backphosphorylation [26]. CCAR cells (2×10^6 per 35 mm plastic culture dish, Corning) were incubated in a low-K^+ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-Tris, pH 7.4) in the presence or absence of db-cAMP (5 mM) or forskolin (100 μM) for various periods at 37°C. The cells were solubilized with 3% CHAPS in NEHDPF (75 mM NaCl, 50 mM Na-phosphate, 2.5 mM EDTA, 20 mM NaF, 50 mM HEPES-Tris, and the protease inhibitors, pH 7.4) and incubated with either CR2 or control rabbit IgG for 2 h at 4°C. The antigen-antibody complex was absorbed onto protein A-Sepharose, washed three times by centrifugation with the same solution and three times with phosphorylation buffer (0.1% CHAPS, 6 mM EGTA, 6 mM $MgCl_2$, and 50 mM HEPES-Tris, pH 7.4), then incubated in phosphorylation buffer containing 2 μM [γ-^{32}P]ATP (5 μCi) and the catalytic subunit of PKA (16 U) for 10 min at 30°C. The reaction was stopped by adding cold 0.3% CHAPS in NEHDPF and the phosphorylated proteins were analyzed as described above.

2.6. *Electrophoresis*

Proteins were separated by SDS-PAGE as described previously [23] using a linear 4–12% acrylamide gradient gel (Tefco).

2.7. *Electrophysiological measurements*

The membrane current was measured by means of the patch-clamp method [27] using a pipette solution containing 65 mM CsCl, 60 mM CsOH, 55 mM aspartic acid, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP, 5 mM phosphocreatine and 10 mM HEPES at pH 7.4 with CsOH. Cells were seeded in the chamber mounted on the inverted microscope, and perfused with Ca^{2+}-free Tyrode's solution (136.9 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM glucose and 5 mM HEPES at pH 7.4 with NaOH). When the whole cell configuration was achieved, the solution was replaced with BA^{2+}-recording solution (136.9 mM tetraethyl ammonium chloride, 5.4 mM CsCl, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10 mM $BaCl_2$ and 5 mM HEPES at pH 7.4 with tetraethyl ammonium hydroxide) to obtain a high enough amplitude of I_{Ba} through the DHP-sensitive Ca channels. We used an experimental set-up connected on line to a computer (Atari MEGA ST-4) to supply command potentials and to acquire data immediately. A patch clamp amplifier, EPC-7 (List electronics, DA-Eberstadt, Germany) was used to record currents. Currents were low pass filtered (1 kHz) by an eight-pole Bessel filter, viewed on a CRT computer terminal, and recorded on a hard disk for further analysis.

2.8. *Materials*

The sources of drugs and chemicals were as follows: the catalytic subunit of type I cAMP-dependent protein kinase, Sigma; [^{3}H]PN200-110 and [γ-^{32}P]ATP, New England Nuclear; peroxidase-conjugated goat anti-rabbit IgG, Zymed Laboratories; protein A-Sepharose CL-4B, Pharmacia, LKB Biotechnology; db-cAMP and 8-bromo-cAMP, Yamasa; digitonin, Wako Pure Chemical Industries; CHAPS, Dojin; ECL Western blotting detection reagent, Amersham; and all other chemicals, Wako, Sigma, or Bio-Rad Laboratories.

3. RESULTS AND DISCUSSION

A polyclonal antibody, CR2, was generated by immunizing a rabbit with the C-terminal region of the α1 subunit of the rabbit DHP-sensitive cardiac Ca channel expressed in *E. coli*. As shown in Fig. 1, CR2 im-

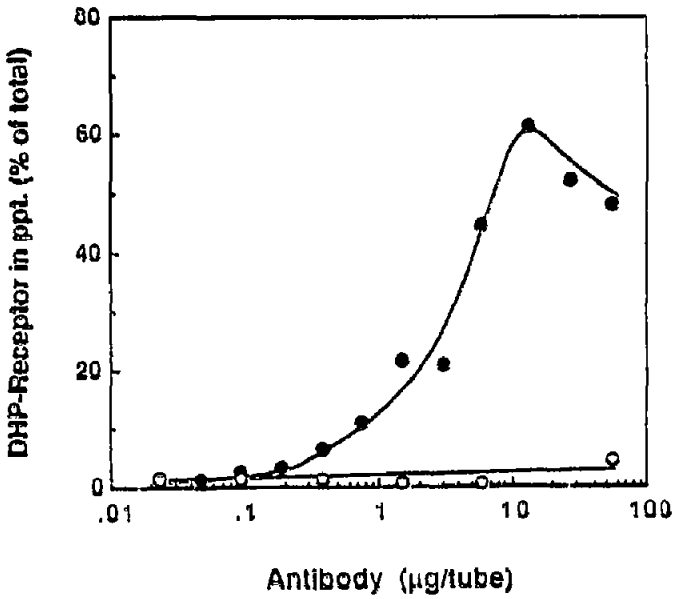

Fig. 1. Immunoprecipitation of [^{3}H]PN200-110-labeled cardiac calcium channels by CR2. The [^{3}H]PN200-110-labeled calcium channels solubilized and partially purified from rabbit heart were incubated with the indicated amounts of CR2 (●) or control rabbit IgG (○), then the antigen-antibody complexes were precipitated by absorption onto protein A-Sepharose. The radioactivity recovered in the precipitate was expressed as a percentage of the total counts added.

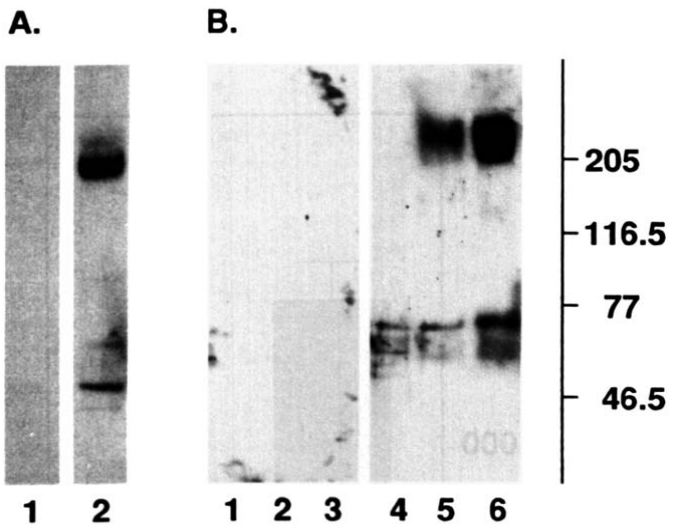

Fig. 2. Immunoblotting of α1 subunit of rabbit heart and CCAR membranes with CR2. Membrane proteins were separated by SDS-PAGE, transblotted to nitrocellulose and immunostained with control IgG (A, lane 1 and B, lanes 1–3) or CR2 (A, lane 2 and B, lanes 4–6) at a concentration of 10 μg/ml. (A) Rabbit heart (4 μg per lane). (B) Lanes 1 and 2, CHO cells (20 μg per lane); lanes 2 and 5, CCAR2823 (10 μg per lane); lanes 3 and 6, CCAR3217 (4 μg per lane). The migration positions of molecular mass standards ($M_r \times 10^{-3}$) are indicated on the right.

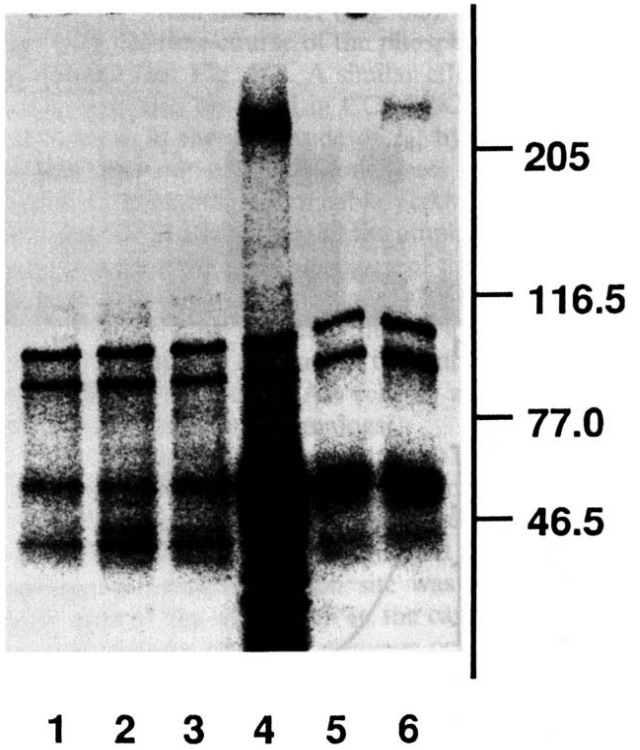

Fig. 3. Phosphorylation of the cardiac α1 subunit from the rabbit heart and CCAR cell by PKA in vitro. The α1 subunits were solubilized from CHO cells (lanes 1 and 2), CCAR3217 cells (lanes 3 and 4) and rabbit hearts (lanes 5 and 6), and immunoprecipitated with either control IgG (lanes 1, 3 and 5) or CR2 (lanes 2, 4 and 6). The immunoprecipitates were incubated with [γ-^{32}P]ATP and a catalytic subunit of PKA for 10 min at 30°C. The phosphorylated proteins were analyzed by SDS-PAGE then image analysed. The migration position of the molecular mass standards ($M_r \times 10^{-3}$), are indicated on the right.

munoprecipitated more than 60% of [^{3}H]PN200-110-labeled Ca channel solubilized from rabbit cardiac membranes.

A polypeptide of 200 kDa was identified as the major antigen in the rabbit cardiac membranes by immunoblotting with CR2 (Fig. 2A). In addition to the 200 kDa band, a faint band at 250 kDa was detected by blotting. Since the molecular mass of the α1 subunit of the rabbit cardiac Ca channel deduced from the amino acid sequence is 242,771 [17], these results suggest that a large part of the α1 subunit in the cardiac membrane preparation is partially proteolysed. In order to confirm this assumption, we prepared two stable transformed CHO cells (CCAR2823 and CCAR3217) using an expression plasmid which carries the entire protein-coding sequence of the rabbit cardiac α1 subunit. As shown in Fig. 2B, a polypeptide of 250-kDa was identified by immunoblotting in the transformants, but not in untransformed CHO cells. There was a marked difference in the amount of expression between these two transformants and much more 250-kDa protein was expressed in CCAR3217 than in CCAR2823 cells.

To examine the phosphorylation, the α1 subunit was solubilized from the membrane fractions of rabbit heart and the two transformants, immunoprecipitated with CR2 and incubated with a catalytic subunit of PKA and [γ-^{32}P]ATP. As shown in Fig. 3, only the 250-kDa form of the α1 subunit from rabbit cardiac membranes was phosphorylated by PKA and no incorporation of P_i was detected in the 200-kDa component. These results are in good agreement with those previously obtained by us [14] as well as by Chang and Hosey [15] using chick heart. The 250-kDa polypeptide expressed in CCAR2823 and CCAR3217 cells was also phosphorylated by PKA and this protein was not detected in non-transformed CHO cells. These results clearly showed that the α1 subunit of cardiac Ca channels can be phosphorylated by PKA and that a large part of the α1 subunit in the cardiac membranes lost the phosphorylation site, probably due to partial proteolysis. The partial cleavage of the α1 subunit similar to that found in the present study occurs in skeletal muscle [28]. The molecular mass of a large portion of the α1 subunit from the T-tubular membrane fraction was 170 kDa [5], which was smaller than that deduced from the amino acid sequence (212,018) [6] and that expressed in L cells (195 kDa) [29]. A minor component of the α1 subunit having a molecular mass of 212 kDa was identified in T-tubular membranes using an antibody against the C-terminal peptide of the skeletal α1 subunit [28, 30].

In order to determine whether endogenous PKA can phosphorylate the α1 subunit incorporated in the cellular membrane, cAMP-dependent phosphorylation of the α1 subunit was studied using CCAR cells and

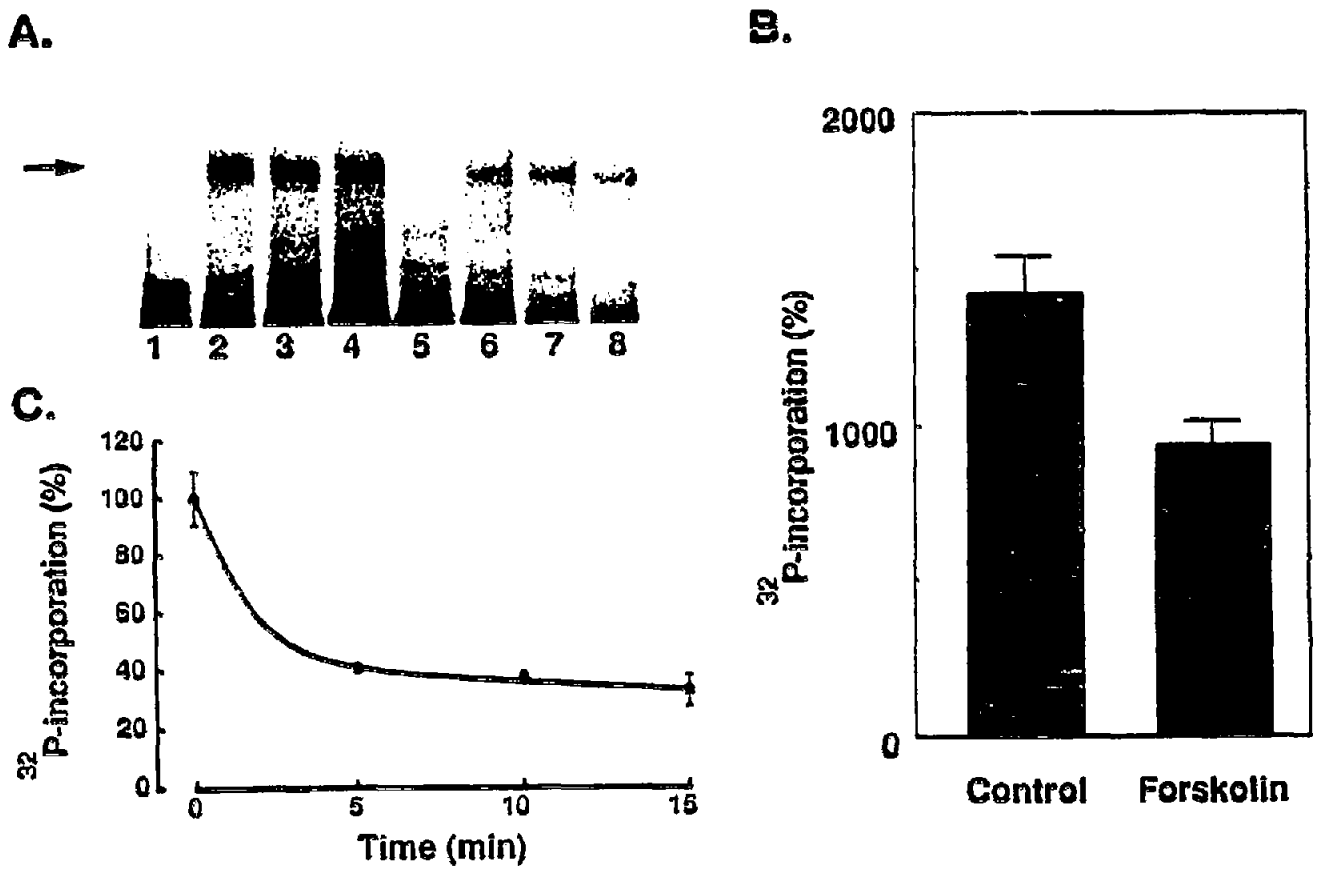

Fig. 4. Cyclic AMP-dependent phosphorylation of the α1 subunit of CCAR3217 cells in vivo. (A) CCAR3217 cultured on 35 mm plastic dishes were treated with 100 μM forskolin (lane 5 to 8) or DMSO (lanes 1–4) for 10 min. After solubilizing the cells with CHAPS, the α1 subunit was immunoprecipitated with CR2 (lanes 2–4 and 6–8) or control rabbit IgG (lanes 1 and 5), then phosphorylated with [γ-^{32}]ATP and PKA. The phosphorylated proteins were separated by SDS-PAGE and analyzed with a Fuji Bioimage-analyzer. The migration position of the 250-kDa phosphoprotein is indicated by an arrow. (B) Results shown in panel A are quantified and the radioactivity incorporated into the 250-kDa bands is expressed in arbitrary units. (C) CCAR3217 cells were treated with 5 mM db-cAMP and solubilized with CHAPS in NEHDPF at the indicated times. The α1 subunit was immunoprecipitated with CR2, phosphorylated with [γ-^{32}]ATP and PKA, and the radioactivity incorporated into the α1 subunit was determined as described above.

backphosphorylation. The CCAR3217 cells were treated with or without forskolin and the α1 subunit was solubilized, immunoprecipitated with either CR2 or control rabbit IgG and incubated with PKA and [γ-^{32}P]ATP. As shown in Fig. 4, the incorporation of ^{32}P into the 250-kDa protein was suppressed by 35% by forskolin. This suppression was also observed after treatment with the membrane permeable cAMP analog, db-cAMP (Fig. 4C). These results indicated that the 250-kDa protein expressed in the CCAR3217 cells was phosphorylated in vivo probably by endogenous PKA in response to an elevated intracellular cAMP concentration. As shown in Fig. 4C, phosphorylation occurred very rapidly and ceased within 5 min after the treatment.

DHP-sensitive Ca channels in cardiac muscles are activated by phosphorylation with PKA in β-adrenergic stimulation [1]. In order to determine whether PKA-mediated phosphorylation of the 250-kDa form of α1 subunit could affect the Ca channel function, we investigated the electrophysiological properties of the CCAR cells. Under our experimental conditions, we did not observe I_{Ba} in CHO cells. In CCAR3217, the depolarizing pulses from the holding potential at −60 mV induced inward currents (Fig. 5). The activation threshold was −20 mV and the peak amplitude of the current was obtained at +20 mV. The current was completely inhibited by 10 μM nicardipine (inset of Fig. 5B). Although these characteristics of the inward current recorded from CCAR were similar to those of the DHP-sensitive Ca channel current recorded in the heart [31], the inactivation time course of the current was very slow as shown in Fig. 5A. The inactivation time constant was 1.11 s at +20 mV. However, the voltage dependency of the steady state inactivation of I_{Ba} in CCAR was similar to that in the heart [31]. The apparent inactivation of the current was observed at below −60 mV and 50% inactivation was obtained at −32 mV (data not shown). An

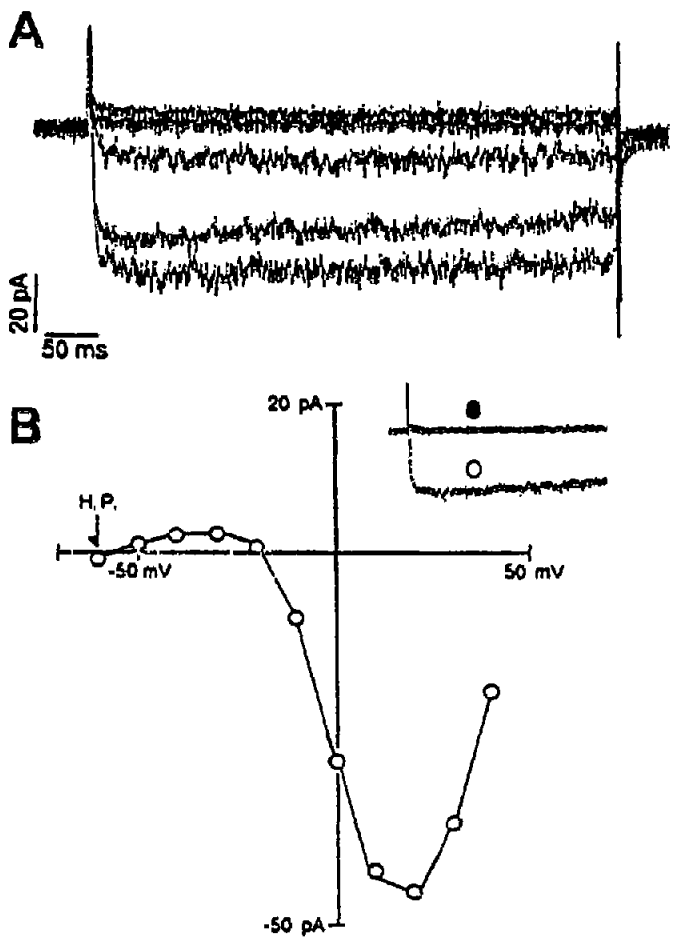

Fig. 5. The Ba current recorded from CCAR3217. (A) The family of currents obtained by depolarization to −50, −30, −10, +10 and +30 mV for 500 ms from a holding potential of −60 mV. (B) The current-voltage relationship of I_{Ba}. The current amplitude measured at 80 ms after the onset of a depolarizing pulse is plotted against the membrane potential. The inset shows superimposed current records at +30 mV obtained in the absence (○) and the presence (●) of 10 μM nicardipine.

I_{Ba} of similar characteristics was also obtained in CCAR2823, however, the amplitude of the current was significantly lower than that in CCAR3217, possibly due to the lower expression of the α1 subunit in CCAR2823 cells (see Fig. 2). These results are consistent with those previously reported [17, 32–34] indicating that the α1 subunit plays a central role in the DHP-sensitive Ca channel function.

Fig. 6A and B shows the increasing effect of db-cAMP on I_{Ba}. The effect was most remarkebly observed in this particular experiment. In the presence of 200 μM db-cAMP, the amplitude of I_{Ba} elicited by depolarization to +20 mV from a holding potential of −60 mV, was increased from −47.1 pA to −119.0 pA. The inactivation time constants of I_{Ba} at this potential were 1.29 s and 1.53 s, in the absence and the presence of the drug, respectively. The amplitude of I_{Ba} at +20 mV gradually increased after adding db-cAMP, and reached a steady level about 5 min thereafter (Fig. 6C). This was consistent with the time course of the phosphorylation of the α1 subunit (see Fig. 4C). A similar effect of db-cAMP on I_{Ba} was also observed in CCAR2823. We observed an increase in the amplitude of I_{Ba} by db-cAMP in 8 experiments out of 19 in both types of CCAR cells. Another membrane-permeable cAMP analog, 8-bromo-cAMP, also increased the amplitude of I_{Ba} in 14 experiments out of 25. The degree of the increase of the current varied among cells, and it was usually about 20%. Neither drug increased the I_{Ba} at concentrations below 100 μM. The decrease in the amplitude of I_{Ba} was not observed in both CCAR cells by adding the membrane-permeable cAMP analogs.

The present studies showed that: (i) the α1 subunit of cardiac Ca channel could be phosphorylated by PKA in vitro and in vivo and that the Ca channel was activated by phosphorylation, and (ii) that the segment containing the phosphorylation site was cleaved from a large part of the α1 subunit in the cardiac membrane. It is not known when the cleavage occurs and at least three possibilities could be considered for the structure of the α1 subunit in cardiac cells: (1) the 250-kDa form of the α1 subunit is predominant in living cardiac cells and the partial degradation resulting in the 200-kDa polypeptide due to the proteolytic cleavage occurs during the isolation of the cardiac membrane; (2) the C-terminal segment of most of the α1 subunit is cleaved but still associated with the 200 kDa polypeptide and functions as a subunit. The α2 and δ subunits of skeletal muscle Ca channels are encoded by the same gene, the protein product of which is proteolytically processed to yield the disulfide-linked α2 and δ polypeptide [7,28]; (3) the C-terminal segment containing the phosphorylation site(s) was lost due to the partial proteolysis in cardiac cells. In the third possibility, the phosphorylation of the C-terminal segment could not be involved in the β-adrenergic-mediated Ca channel regulation. Thus, the identification of the α1 subunit in cardiac cells should be studied for further understanding of the regulatory mechanisms of the cardiac Ca channel function.

The amplitude of I_{Ba} observed in considerable numbers of CCAR cells is much lower than that in cardiac myocytes. Furthermore, the extent of the activation by cAMP-dependent phosphorylation in the CCAR cells was also less than that in the heart. Is has been shown that I_{Ba} expressed by injecting the α1 message into *Xenopus* oocytes and L cells was increased remarkably by the coexpression of ß and γ subunits of the skeletal muscle Ca channel [17, 32–34]. Northern blots have shown that the ß subunit homologous to that of skeletal muscle was expressed in cardiac muscle [34]. Thus, the formation of a multisubunit complex might be necessary for expressing the activation of DHP-sensitive Ca channels. Further studies, including the coexpression of the other subunits are necessary to obtain a final conclusion.

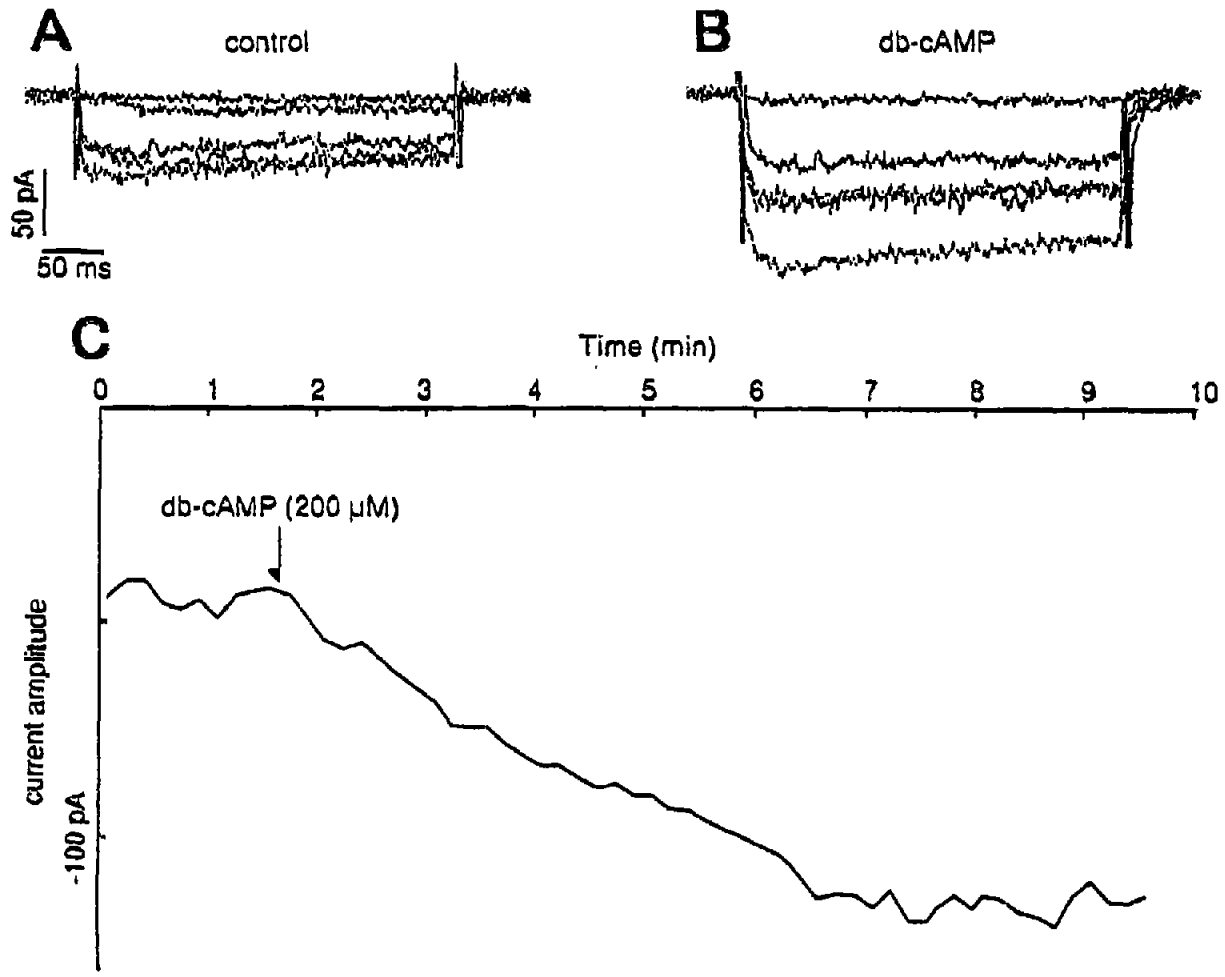

Fig. 6. The effect of db-cAMP on I_{Ba} in CCAR3217. (A) Currents in the absence of db-cAMP recorded by depolarization to −40, −20, 0, +20 and +40 mV for 500 ms from a holding potential of −60 mV are superimposed. (B) Currents in the presence of db-cAMP (200 μm) recorded at same membrane potentials as those in A are superimposed. (C) The time course of the effect of db-cAMP on the amplitude of I_{Ba}. The amplitude of I_{Ba} measured at 80 ms after the onset of the pulse is plotted against the time.

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